THE UNIVERSITY OF ALBERTA

Autotransplantation of Dispersed Pancreatic Tissue Fragments in the Dog

by

Kim F. Duncan

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Autotransplantation of Dispersed Pancreatic Tissue Fragments in the Dog submitted by Kim F. Duncan in partial fulfilment of the requirements for the degree of Master of Science in Experimental Surgery.

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(Supervisor)

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April 22, 1986

Dedicated to Valerie, Cameron and especially to Brett,

whose birth delayed and inspired completion of this

manuscript

Autotransplantation of Dispersed Pancreatic Tissue Fragments in the Dog

Abstract

Type I or insulin-dependent diabetes mellitus and its complications result from deficient insulin production resulting in altered carbohydrate metabolism and a progressive microangiopathy.

Pancreatic islet transplantation restores glucose homeostasis by replacing functional, insulin-producing B cells. Intrasplenic autotransplantation has evolved as a successful model of pancreatic islet transplantation, but the optimal technique for preparation of the pancreatic tissue has not been established.

A simplified method of preparing dispersed pancreatic tissue fragments incorporating hand-mincing and limited collagenase digestion was studied. Efficacy was assessed by comparing the clinical and biochemical status of animals that received intrasplenic autotransplants of prepared tissue with normal and apancreatic control animals.

Normal intravenous glucose tolerance test (IVGTT) and insulin response curves were obtained preoperatively. Total pancreatectomy was performed in 21 mongrel dogs weighing 15-25 kg. Seven dogs received no further therapy and served as apancreatic controls (APC). The remaining animals were autotransplanted by direct injection of prepared tissue into the splenic pulp. In one group

(n=7), tissue was prepared by hand-mincing followed by collagenase digestion at 37° for 10 minutes (CD). In the second group (n=7), hand-mincing was used without collagenase digestion (NCD). Insulin (0.2-0.5 U/kg) was given for 12 days post-operatively to all transplanted animals. The animals' weight, fasting serum glucose levels and survival were recorded. IVGTT's were performed two and six weeks post-transplant. The glucose decay constant (k value) was calculated for each group. Insulin response curves were obtained two weeks after transplantation.

There was no operative mortality. Intrasplenic injection produced no significant complications. Total pancreatectomy produced a characteristic diabetic state with a mean survival of 15 ± 4.4 days. NCD animals survived 24 ± 4.2 days and CD animals survived 43 ± 11 days, the latter significantly longer than the APC group ($p < 0.05$). Mean IVGTT curves, k values and insulin response curves for NCD and CD animals were not different from the APC group, however. Transient normoglycemia was present in two CD animals for 13 and 48 days but IVGTT results and k values were not normal.

Total pancreatectomy in the dog produced a severe diabetic state with rapid deterioration. Although transient normoglycemia was achieved in two animals receiving collagenase-digested tissue, neither method of preparation employed in this experiment was satisfactory. Collagenase appears to benefit tissue processing but further evaluation of its application to the preparation of pancreatic tissue fragments is required.

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Autotransplantation of Dispersed Pancreatic Tissue Fragments in the Dog

I.. Introduction

In reporting to the United States Congress in 1975, the National Comission on Diabetes stated that diabetes mellitus was the fifth leading cause of death from disease (11). Approximately five percent of the population had diabetes, and their life-expectancy was two-thirds that of the general population. The incidence of diabetes was noted to be increasing by six percent per year and the number of diabetics in the population was expected to double in 15 years.

Diabetes mellitus is an endocrine disorder characterized by hormone-induced metabolic abnormalities, neurovascular lesions and long-term complications (18). Diet therapy and exogenous insulin administration have profoundly altered the short-term course of diabetes. However, significant morbidity and mortality are now well recognized as resulting from major complications developing 10 to 20 years after the diagnosis has been made (18). The elevated blood glucose level that appear to be the major contributing factor in the development of the lesions which appear in patients with long standing diabetes mellitus (28). Strict control of blood glucose levels by administering multiple injections of insulin daily will slow the progression of microvascular lesions

(61) but in clinical practice, even when so-called tight control is maintained, diabetics often demonstrate wide fluctuations in blood glucose levels that do not occur in non-diabetic individuals (28,61). New modes of therapy are required in an attempt to prevent, arrest or delay the development of the late complications of diabetes mellitus. One approach has been the transplantation of pancreatic islet tissue.

II. Review of Pancreatic Transplantation

Pancreatic transplantation as a method of treating diabetes was first suggested by Szobolew in 1902 (77). However, the first pancreatic transplants were performed in experimental animals by Hedan in 1893 (77). Portions of duct-ligated pancreas were heterotopically transplanted in dogs after total pancreatectomy and it was found that diabetes did not develop until the transplants were removed. Hedan hypothesized that an internal secretion was produced by the transplanted portion of pancreas that prevented diabetes, and this secretion was not related to, or dependent upon, the obvious external excretion of the pancreas. Laguesse subsequently proposed that the yellow clusters of cells, first described by Langerhans in the rabbit pancreas in 1869, were responsible for the internal secretion postulated by Hedan (79). Laguesse called the cell clusters "islets of Langerhans".

The concept of transplantation was almost completely forgotten when insulin was isolated from the canine pancreas by Banting and Best in 1921 (4), followed by a new era in the therapy of diabetes mellitus when long acting protamine zinc insulin was developed for commercial production by Hagedorn in 1936 (79). During World War II, observations made by Loubatieres led to the development of sulfonyl urea derivatives which could be given orally to stimulate the pancreas to increase insulin output. By 1955, clinical trials on diabetic patients had been undertaken and oral hypoglycemics were becoming a significant adjunct in the therapy of diabetes mellitus (79).

Insulin has a significant effect on diabetes mellitus by controlling symptoms, preventing ketoacidosis, and prolonging life. However, as diabetics lived longer, previously unrecognized complications were found to develop affecting the eye, kidney, cardiovascular and nervous systems. It has been proposed that the development or progression of the late complications of diabetes mellitus can be ameliorated by precise regulation of carbohydrate metabolism and that this can best be achieved "through the homeostatic control of metabolism by endogenous release of insulin from transplanted islets or pancreas" (51). While the question of whether or not normalization of carbohydrate metabolism will prevent the development of late complications has not been completely resolved, several observations can be made which support the hypothesis that the lesions associated with diabetes are secondary to abnormal metabolism. First, several long-term follow-up studies have shown that a relationship exists between the duration of the disease, control of blood sugar levels and the development of retinopathy and nephropathy (51). Second, these lesions develop in patients with diabetes secondary to other disease states affecting the pancreas, for example, hemochromatosis (51). Third, nephropathy and retinopathy develop after diabetes is induced in laboratory animals, and these lesions are similar to those appearing in human diabetics (23,79). Fourth, animal studies have shown that insulin therapy or transplantation of pancreatic islets, which reduce hyperglycemia, can prevent or minimize the late or secondary lesions of the eye, kidney and nerve (23,50,70,75). Finally, renal transplant experiments in isogeneic

rats have demonstrated that kidneys transplanted from diabetic to normal animals show resolution or arrest of the diabetic changes, whereas normal kidneys transplanted to diabetic rats develop the characteristic histologic lesions of diabetes mellitus (45).

These observations have provided the impetus and justification for continuing investigative efforts to develop a safe, efficient method of transplanting pancreatic islets. While the ultimate cure for diabetes may be found in methods designed to correct or bypass specific defects in the diabetic B cell, replacement of defective B cells by transplantation of normal islets can provide a direct and immediate solution to the problem (41).

A. Whole Organ Pancreatic Transplants

Initial efforts at replacing defective B cells attempted to transplant whole or segmental organ grafts. Houssay, in 1927, transplanted the pancreas to the neck of a dog and demonstrated short-term reduction of hyperglycemia (51). Numerous techniques have been reported since that time and all attest to the complications associated with whole organ pancreatic transplants, including vascular thrombosis, pancreatitis, graft rejection and difficulty in establishing control of the exocrine secretions from the pancreatic duct (46). The first systematic trials of human pancreas transplantation began in 1966 when Kelly, Lillehei and associates performed combined renal and whole organ pancreatic transplants (6,29). By 1974, Lillehei and associates had demonstrated the effectiveness of whole organ pancreatic transplants in diabetic patients using a pancreatico-

duodenal graft, but the morbidity and mortality were high (22). Sepsis, graft rejection, anastomotic breakdown, fistula formation and hemorrhage were encountered. Control of the exocrine secretions became a primary concern because the unregulated enzymatic action of the secretions was thought to be responsible for most of the complications (10,71). A variety of techniques were studied including irradiation of the gland, administration of 5-fluorouracil or methylprednisolone, duct ligation, cutaneous duodenostomy and drainage by an intraperitoneal Roux-en-Y loop (43,51,78). It became apparent that the inclusion of any portion of the duodenum was associated with the highest incidence of complications, including rejection (76). By eliminating the duodenum from the graft and with the knowledge that the tail of the pancreas contains the largest number of islets per unit of tissue (25,57), the next logical step was to transplant the distal portion of the gland based on a vascular pedicle made up of the splenic artery and vein.

B. Vascularized Segmental Pancreatic Graft

As with whole organ transplants, the major technical problem with vascularized segmental pancreatic grafts has been the management of the exocrine secretions. Four basic manoeuvers have been used to control the pancreatic secretions: duct ligation, duct obliteration, closed duct drainage and open duct drainage.

Ligation of the main pancreatic duct was advocated by Kelly and Lillehei in their early work with whole pancreas transplants (29). Duct ligated grafts have been successfully transplanted in animals

and humans, but complications such as pancreatitis, fistulae, loculated fluid collections, and sepsis, developed in almost all cases (72).

There has also been controversy regarding the late effects of pancreatic duct ligation on islet cell function. Dragstedt reported in 1943 that diabetes frequently developed in dogs as a result of extensive degeneration of the pancreas after occlusion of the pancreatic duct (17). In 1969, Idezuki and colleagues demonstrated that severe atrophy and fibrosis of exocrine tissue secondary to ductal ligation could finally lead to endocrine insufficiency (26). A significant number of the animals that received duct-ligated pancreatic grafts demonstrated latent or overt diabetes. They postulated that the atrophied, fibrotic pancreas maintaines the ability to secrete insulin but that there was diminished blood flow across the islets in the fibrotic pancreas after duct ligation. The reduced blood flow was insufficient to remove adequate quantities of insulin from the pancreas in response to a hyperglycemic challenge (26). Other investigators reported the development of hyperglycemia six to eight months after autotransplantation of canine pancreatic segmental grafts; shorter observation periods did not identify endocrine insufficiency although atrophy and fibrosis were present. Idezuki concluded that while the human pancreas may be less susceptable to fibrosis after transplantation, particularly when rejection is controlled, free drainage of exocrine secretion should be used to prevent the fibrosis and possible secondary deterioration of endocrine function (26).

A contrasting view was presented by investigators from Georgetown University (69). Morphological findings were studied in duct-ligated isografts, allografts and longstanding allografts in conditioned hosts. Minimal fibrosis, an absence of inflammatory cells and indefinite survival characterized the isografted pancreatic tissue while both types of allograft demonstrated varying degrees of pancreatitis, dense fibrosis and islet destruction. It was concluded that the pancreatitis that occurred in duct-ligated pancreatic allografts was not a sequel of duct ligation, but rather, a result of rejection, and that fibrosis in response to duct ligation does not have a detrimental effect on islet cell function (69).

Duct obliteration by injecting Neoprene into the main pancreatic duct at the time of surgical removal from the donor was advocated by Traeger and Dubernard (16,74). Neoprene is a synthetic polymer that, when injected into the pancreatic duct, fills the duct and the acini of the pancreas. The material then hardens and prevents release of the enzymes. However, it is sometimes difficult to completely fill all acini and small loculations of enzyme-rich fluid can collect and become sites of active tissue digestion or infection. Neoprene also incites a reactive, progressive sclerosis about the obliterated duct and most of the grafts performed by Traeger and Dubernard ceased to function after one to eight months due either to the fibrotic reaction to the Neoprene or from graft rejection (15). Cyanoacrylate tissue adhesive and silastic have also been employed to obliterate the pancreatic duct system, and a lower incidence of complications has been reported with each technique (48,66).

The question of the long-term effects of duct occlusion on islet cell function in segmental pancreatic transplants has not been fully answered, but these potential complications may be avoided if the grafts can be successfully transplanted without obliterating the ducts. Closed drainage techniques direct exocrine secretions into a hollow viscus or externally to the skin surface. Creation of an external fistula has numerous disadvantages (46) and most investigators have concentrated on internal drainage methods. Aquino and associates maintained a rim of papillary mucosa about the duct orifice to facilitate construction of a duct-jejunostomy (2). Dickerman constructed a retroperitoneal limb of jejunum and, during a second surgical procedure, transplanted a segmental pancreatic graft, draining the duct into the sequestered limb of jejunum (12). Anastomotic breakdown and leakage with subsequent fluid accumulation and sepsis remained as significant complications. One technique of internal drainage, which has produced continued graft function and normoglycemia in dogs for more than three years, was developed by Gliedman and Gold (20). An anastomosis was made between the pancreatic duct and the adjacent distal end of ureter, allowing pancreatic secretions to drain into the bladder. Initial studies showed no evidence of pancreatitis, pancreatic atrophy or urinary tract injury, and histological examination of the bladder was normal. The pancreatico-ureteral anastomosis, performed over a polyethylene stent, usually healed within one week and patency was reported as excellent. This technique has been applied clinically with some success (21).

Kyriakides demonstrated that free drainage of pancreatic secretions into the peritoneal cavity in pigs was well tolerated and did not lead to significant complications (39). Activated pancreatic enzymes are responsible for the pathological process producing leakage, bleeding and tissue digestion. However, this technique was based on the fact that trypsinogen, the major pro-enzyme in pancreatic juice, was not activated in the absence of enterokinase, normally derived from gut mucosa. The exocrine secretions from the pancreas were allowed to drain from the unligated, unobliterated pancreatic duct and were reabsorbed by the peritoneum. Kyriakides and Sutherland subsequently applied the open drainage technique in dogs and found that pancreatic grafts with unligated, non-anastomosed ducts maintained good endocrine function over several months of observation (40).

Immediately vascularized whole or segmental pancreatic grafts have had only limited success in restoring blood glucose levels to normal in experimental (2,6,40,46) clinical studies (72). Difficulty still persists in controlling immune rejection and maintaining ex vivo organ viability to permit selection and preparation of transplant recipients. As a result, a great deal of interest has been directed to another mode of pancreatic transplantation, utilizing free grafts of islet tissue.

C. Transplantation of Pancreatic Islets

Initial attempts at pancreatic islet transplantation incorporated implantation of tissue fragments in various sites in experimental

animals, including the testis, the anterior chamber of the eye and pockets in muscle and subcutaneous tissue (9). The anterior chamber of the eye isolated allotransplanted tissue from host defences, but the small amount of tissue that could be accomodated by this site was insufficient to maintain normoglycemia. Other sites such as subcutaneous or muscle pockets permitted implantation of larger amounts of tissue, but immune rejection rapidly terminated function of transplanted tissues. There also appeared to be wide variation in the efficacy of tissues transplanted at various sites demonstrating variable tolerance to the transplanted tissue. Attempts at implanting adult pancreatic tissue fragments in muscle pockets were unsuccessful because of damage to host and donor tissues by the associated exocrine enzymes (7).

Investigators were therefore confronted by three problems in attempting to transplant pancreatic islets. Immune rejection was, and continues to be, the pre-eminent problem (71). Islets have been demonstrated to be immunogenic, and allografts of islet tissue are rejected rapidly (5,19,33,41,65,67). Using conventional methods of immunosuppression, limited success has been achieved with allograft islet transplantation in laboratory animals (71). Much of the experimental work involved in solving the less formidable problems associated with islet transplantation has been performed using isogeneic strains of mice or rats, and autotransplants in larger animals such as the dog or monkey, in order to bypass the phenomenon of rejection.

Determination of the optimal site of implantation is the second problem encountered. Insulin from the pancreas is normally secreted

into the portal venous system, draining to the liver and the concentration of insulin in the portal venous blood is ten times the concentration in peripheral venous blood. Lacy, in a discussion of potential sites for tissue implantation, concluded that the site should be chosen "to ensure portal drainage and access of high insulin concentrations reaching the liver" (3). Kemp et al compared the function of isolated islet isografts transplanted subcutaneously, intraperitoneally, directly into the portal vein or into the posterior vena cava of diabetic rats. The portal site of injection resulted in embolization of islets to the portal tracts of the liver, and this site was found to be superior to all other sites tested (30,31). The spleen also drains into the portal venous system and has been shown to be an effective site for implantation (71). In particular, the spleen tolerates a larger volume of tissue than the liver, as well as providing a rich vascular bed to nourish transplanted tissue (38).

For pancreatic islet transplantation to correct the metabolic abnormalities of diabetes successfully, a sufficient number of viable islets must be transplanted. The source and technique of preparing islets is the third major problem associated with pancreatic islet transplantation. Using fetal, neonatal or adult pancreatic tissue, a variety of techniques have been developed to harvest viable islets.

As in whole or segmental organ transplantation, initial experiments demonstrated that control of the effects of the exocrine portion of the pancreas appeared to be critical (35). Efforts were directed primarily at separating the endocrine and exocrine portions

of the gland. One of the first effective methods was reported by Moskalewski in 1965, in which collagenase, an enzyme complex derived from a bacterium, was used to partially digest pancreatic tissue and liberate islets in the guinea pig (63).

Lacy and Kostianovsky adapted this technique into a method for isolating intact pancreatic islets from the normal rat pancreas (42). Mechanical disruption of the exocrine tissue was achieved by injecting a salt solution under pressure into the common bile duct and pancreatic ducts. After mincing the tissue, controlled collagenase digestion was carried out and isolated islets could be hand-picked from the partially digested pancreatic tissue under the dissecting microscope. A more rapid technique of isolating pancreatic islets after mincing and limited digestion was developed by Lindall and associates, using centrifugation and a Ficoll discontinuous density gradient (47). Ficoll, a high molecular-weight polymer of sucrose, could be used to separate islets, but usually damaged them in the process. In 1973 Scharp and associates reported that insulin release from isolated islets was normal if the Ficoll was dialyzed before its use (68) and collagenase digestion with dialyzed Ficoll separation became the standard islet isolation technique.

In 1972 Ballinger and Lacy reported the first successful transplant of isolated islets in experimental animals (3). Four to 600 isolated adult islets were implanted in the peritoneal cavity of isogeneic rats and reversal of streptozotocin-induced diabetes was demonstrated over a three-month period of observation.

Lacy emphasized the advisability of portal venous drainage of the transplanted islets and Kemp and his colleagues subsequently confirmed that intraportal injection of the isolated islets was the most effective mode of islet transplantation (30).

Isolation techniques were characterized by low islet yield from a single donor pancreas, and multiple organs were required to ameliorate diabetes in the rat, using either adult or neonatal pancreatic tissue. Subsequently, it became obvious that the techniques for islet preparation developed in rats would not work when applied to the more compact pancreases of the pig, monkey and man (35,68). Sutherland noted that most or all of the early grafts in humans failed, probably because of the problems related to preparation of the islet tissue. The average yield of islets from adult human pancreases was less than 5%, from infant human pancreases, 7% (73).

The low yield of islets and poor clinical results have directed attention at the development of ancillary techniques of tissue preparation to produce a larger yield of viable islets. Scharp utilized limited exposure to collagenase during multiple one-minute digestion periods and a stainless steel micropore filter which permitted liberated islets to pass out of the digested tissue mass (68). Another technical modification, incorporated by Downing and associates, was venous distension of the gland prior to mincing (13).

Lacy and Kostianovsky (42) had shown that mechnical disruption by ductal distension improved the islet yield in the rat, but in the dog, ductal distension of the pancreas produces an interlobular

disruption which does not physically dissociate the islets and the surrounding exocrine tissue. To achieve this, intralobular disruption must occur (14). Injection of the arterial system produced excellent intralobular disruption but the islets were severely damaged. Venous injection however, produced the desired effect and increased the islet yield four-fold compared to ductal distension. Venous distension did not appear to damage the islets, and collagenage digestion was also facilitated (68).

An alternative to isolating endocrine tissue from mature pancreas was introduced after studies of fetal and neonatal rat pancreases revealed that developmentally, functional islet tissue appears at a time when acinar tissue is relatively undifferentiated (24,53). Leonard and associates established that neonatal rat pancreas between the ages of 2.5 to 4.5 days had the highest insulin concentration, the largest islet volume-percent and the lowest enzyme concentration of either the fetal or post-natal period (44). Because neonatal pancreatic tissue is not fibrotic compared to adult tissue, it easily disrupts with minimal exposure to collagenase and islet cell damage or loss can be minimized (27,64).

The essential difficulty in preparing isolated islet cells lay in obtaining an adequate number of viable islets which would control hyperglycemia when transplanted. In 1976, Mirkovitch and Campiche demonstrated that complete separation of endocrine from exocrine tissue was unnecessary, and that fragments of pancreatic tissue composed of acinar and islet cells could be successfully autotransplanted in dogs (58). After total pancreatectomy, the horizontal

portion of the pancreas containing the majority of the islet tissue, was prepared by collagenase injection directly into the gland and then chopping into small fragments by hand. The dispersed pancreatic tissue fragments were then autotransplanted by retrograde venous injection into the spleen. All transplanted animals were long-term survivors, and glucose tolerance tests performed during the fifth postoperative week did not differ from a control group of normal dogs. Serum insulin levels rose appropriately (58). The successful function of the transplanted pancreatic tissue was confirmed by splenectomy performed two months after implantation. In all animals, splenectomy was followed by a prompt recurrence of hyperglycemia which persisted until the animals sucumbed four to 13 days later. Histological examination confirmed the presence of normal looking endocrine and exocrine cells in the spleen.

Subsequently, Kolb and associates autotransplanted sedimented pancreatic tissue fragments into either the portal vein or the spleen of dogs (34). In the rat, Amamoo et al had shown that the intrahepatic presence of isolated islets did not significantly alter the microscopic architecture or biochemical profile of the liver (92). However, the use of dispersed pancreatic fragments required the implantation of a relatively large volume of tissue, and severe portal hypertension and disseminated intravascular coagulation frequently occurred in experimental animals (34,56). Kretschmer and associates compared intraportal implantation with intrasplenic injection, either directly or by the arterial or venous systems

in the dog. It was concluded that direct implantation of pancreatic tissue into the spleen was superior when 50 to 120 ml of settled pancreatic tissue were injected (37).

Although fasting blood sugars were normal in animals receiving pancreatic tissue fragment implants, postoperative glucose tolerance tests demonstrated a slower than normal decline in blood sugar indicating latent diabetes (34,35,59). Some investigators have suggested that this may simply reflect an inadequate number of functioning islets that may be corrected by transplanting larger volumes of tissue (34,59). However, impaired islet function due to tissue processing could not be ruled out (34,35).

Kretschmer and Sutherland performed intrasplenic autotransplantation of canine pancreatic tissue fragments and studied islet loss during tissue preparation (35,36). Mechanical mincing resulted in islet loss of up to 50 percent, and increasing periods of collagenase digestion augmented the loss. After incorporating a standard chopping technique, these investigators established that a period of 15 to 20 minutes of collagenase digestion produced the best post-operative glucose tolerance curves, but even using an apparently optimal period of exposure to collagenase, these curves were significantly different from normal controls (36).

By utilizing small fragments of pancreas as the vehicle for transplanting islets, the complications of the techniques of separation of endocrine and exocrine tissue can be avoided. Normoglycemia can be achieved and maintained after intrasplenic implantation of

autogenous pancreatic tissue fragments. Although glucose metabolism is not completely normalized, this model of pancreatic islet transplantation provides a foundation upon which studies may be based in an attempt to refine techniques for the preparation and transplantation of pancreatic islets.

III. Objective

Intrasplenic autotransplantation of dispersed pancreatic tissue fragments has evolved as a successful model of pancreatic islet transplantation. The optimal technique for preparation of the tissue prior to implantation has not been established. Mechanical tissue disruption has been demonstrated to be necessary as an initial step in preparing small fragments of pancreatic tissue (35,68). Exposure to collagenase enhances tissue fragment dispersion, but also contributes to a significant loss of islets (36). The objective of this project was to study the efficacy of a simplified method of tissue preparation incorporating hand-chopping and limited collagenase digestion, which would provide a working model of autotransplantation of canine pancreatic islets.

IV. Experimental Method

A. Study Groups

Twenty-one mongrel dogs weighing 15-24 kilograms were studied in three experimental groups. All experiments were performed at the Surgical-Medical Research Institute of the University of Alberta, where the animals were kennelled in the large animal vivarium and were under the care of the investigators and with advice from a veterinarian, in accordance with the criteria formulated by the Canadian Council on Animal Care. The animals' regular diet consisted of 360 g of meat (Dr Ballard's) and 600 g of Pow R. Pac Burger Bits, and all were permitted unrestricted exercise twice daily.

Prior to being allocated to an experimental group, 12 animals were studied as normal controls. Subsequently, seven dogs underwent total pancreatectomy and served as apancreatic controls (APC group). The remaining 14 animals received autotransplants of dispersed pancreatic tissue fragments by direct injection into the spleen after total pancreatectomy and tissue preparation. In seven, collagenase digestion was performed after hand-chopping (CD group), and in the second transplanted group, also composed of seven dogs, hand-chopping alone was used to prepare pancreatic fragments (NCD group).

B. Pre-operative Glucose and Insulin Determinations

Normal values for glucose tolerance were obtained by performing

pre-operative intravenous glucose tolerance tests (IVGTT) on 12 animals. Dextrose solution (1.5 mg/kg) was injected into a fore-limb vein in awake animals secured in a Pavlov frame. Venous blood samples were collected immediately prior to dextrose injection (time zero) and at subsequent time intervals (10,20,30,60,90,120 minutes). Serum glucose determinations were made by the hexokinase method using an IL Multistat III Analyzer and the normal glucose tolerance curve was plotted. The natural logarithm of mean serum glucose was calculated at 10,20,30 and 60 minutes, according to the method described by Moorehouse et al (62). By plotting these values against time, a linear relationship was demonstrated, and the slope of the line represented the percent by which the serum glucose declined each minute, that is, the glucose decay constant or k value.

Blood samples for insulin levels were drawn concurrently with glucose specimens and all serum for insulin determinations was stored at -20^{o}C until all specimens had been collected. Serum insulin determinations were performed by radioimmunoassay in the Endocrinology Laboratory, Clinical Sciences Building, University of Alberta. The normal serum insulin curve was plotted using values obtained during the pre-operative testing of 12 normal animals.

C. Total Pancreatectomy

Anaesthesia was induced with sodium pentothal (5 mg/kg) and maintained with halothane (0.1-1.0%) and oxygen after endotracheal intubation. Five percent dextrose in normal saline (25 ml/kg)

was infused during the procedure.

The abdomen was opened through a long midline incision and total pancreatectomy was performed after the technique described by Markowitz (49). The pancreas was mobilized by carefully dissecting the vertical portion of the pancreas off the mesenteric border of the duodenum (Plates I & II), preserving the gastroduodenal artery and the duodenal branch of the pancreatico-duodenal artery. The main and accessory pancreatic ducts were ligated as they entered the duodenum. The mesentery suspending the horizontal portion of the pancreas was divided (Plate III) and pancreatic branches of the splenic artery and vein were ligated in continuity and divided, permitting removal of the intact pancreas.

After completion of the experiment the fascia of the linea alba was closed with a continuous absorbable suture (0-Dexon, Davis and Geck). Subcutaneous tissue was approximated with a continuous 3-0 Dexon suture, and skin with a subcuticular suture of 3-0 Dexon. The animal was extubated and 2.0 ml of Pen-Di-Strep antibiotic solution (rogar/STB, London, Ontario) containing 200,000 units of penicillin and 250 mg of streptomycin were injected intramuscularly. The animal was returned to its kennel and allowed to recover from the surgery.

D. Tissue Preparation

The horizontal portion of the pancreas was used in the preparation of tissue fragments. Using sterile technique, the tissue was cut into pieces approximately 5 mm in diameter with a scalpel and

Plate I: Duodenum and greater omentum elevated to demonstrate vertical (▶) and horizontal (▲) portions of the pancreas.

Plate II: Vertical portion of pancreas dissected from medial border of duodenum; careful dissection preserved duodenal vascular supply encountered at the apex of the pancreas (arrow).

Plate III: Horizontal portion of pancreas is suspended in the mesentery forming the dorsal leaf of the greater omentum. Pancreatic branches of the splenic artery and vein, which have to be ligated, are located within the base of the mesentery (arrow).

Plate IV: Using iris scissors, pancreatic tissue was minced by hand in conical glass containers, which were seated in crushed ice to maintain tissue temperature near 4°C.

placed in a conical-shaped glass dish containing 10 ml of cold (4°C) Ringer's lactate solution. The dish and contents were placed in an ice-water bath (0°C) and mincing was accomplished by hand using two pairs of sharp iris scissors (Plate IV). After approximately 10 minutes of chopping, the tissue fragments were washed with 500 ml of cold buffered (pH 7.5) Ringer's lactate solution containing Trasylol (Aprotinin, Miles Pharmaceuticals, Rexdale, Ontario) 500 KIU/ml and glucose 100 mg/dl and centrifuged. In the NCD group the sedimented tissue fragments were then suspended in 30 ml of cold Ringer's lactate and repeatedly injected through a 15-gauge needle to complete the mechanical disruption process. Passage through the needle was repeated until the semi-liquid material could be injected through a 16-gauge needle. After resuspending the tissue in the CD group, collagenase digestion was carried out before injection through the 15-gauge needle. Prior to intrasplenic implantation, the tissue in both groups was washed twice with 500 ml of buffered cold Ringer's lactate, centrifuged, and the volume of settled tissue measured after a small sample of tissue had been taken for histological examination (Plate V & VI).

E. Collagenase Digestion

Collagenase (Sigma, type V, lot number 1090-6930) 1000 units/ml, was prepared by disolving dry crystals in 30 ml of buffered Ringer's lactate and sterilized by ultra-filtration (pore size 0.45 microns).

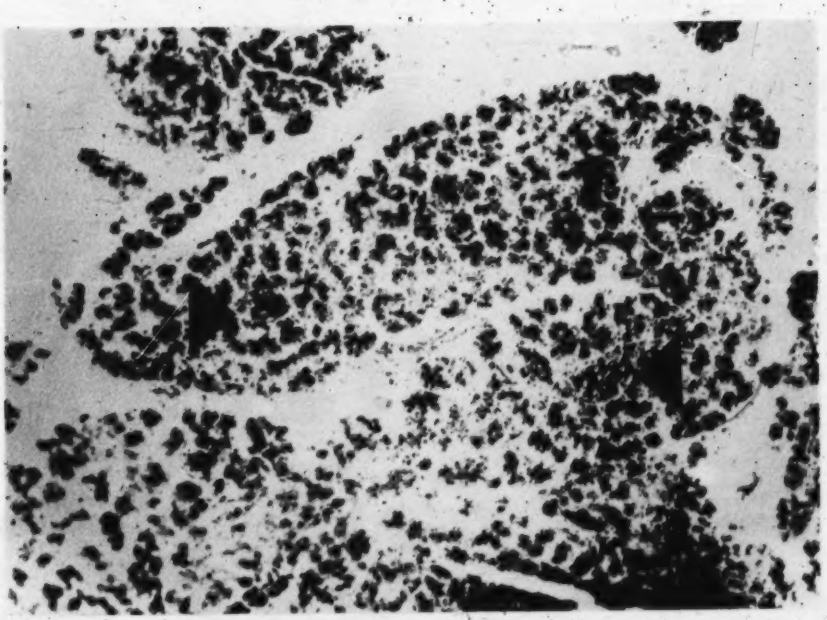

Plate V: Low-power photomicrograph of prepared pancreatic tissue fragments prior to splenic implantation. Islets (arrows) can be identified within the fragments. (H and E x 10)

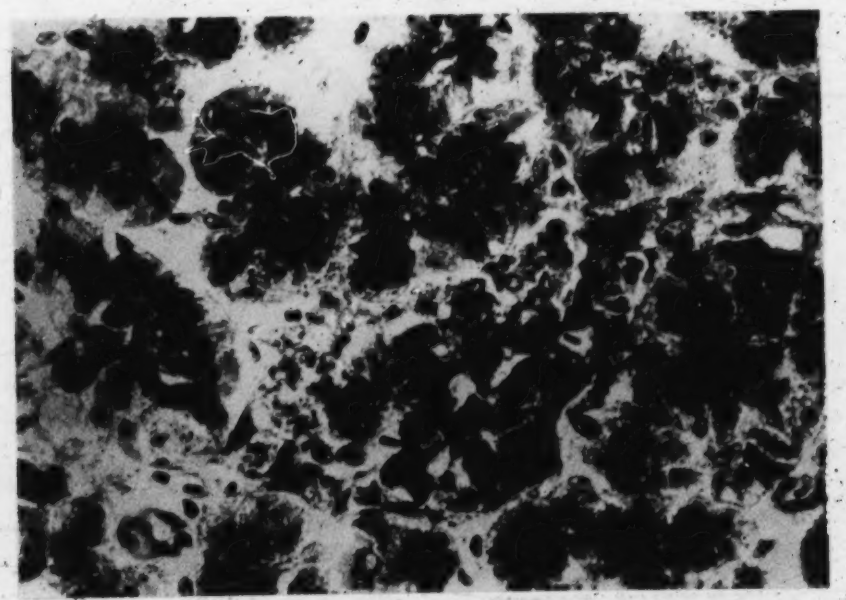

Plate VI: High-power photomicrograph of a single pancreatic tissue fragment prior to splenic implantation. An islet can be identified surrounded by exocrine acini. (H and E x 100)

In the CD group, the washed, centrifuged tissue was placed in a 1000 ml beaker and 1000 units of collagenase per gram of tissue were added. Digestion occurred in a shaking waterbath at 37°C for 10 minutes, and was halted by the addition of 500 ml of cold (4°C) buffered Ringer's lactate. The mixture was centrifuged, the tissue collected, and two additional washes were completed. After re-suspending the tissue fragments in 30 ml of Ringer's lactate, repeated injection through a 15 and a 16-gauge needle was carried out. Final washes were performed prior to splenic implantation.

F. Splenic Implantation

Thirty-nine to 55 ml of settled tissue were available for implantation. After suspending the tissue in 30-50 ml of Ringer's lactate, implantation was performed by direct needle puncture of the margin of the spleen using a 15 or 16-gauge 3-inch needle (Plate VII). Subcapsular and deep intraparenchymal injections were made, and approximately two ml of tissue was implanted at each site. Care was taken to avoid injection near the splenic hilum, to prevent intravascular embolization. Splenic vessels were not occluded during injection. Bleeding from the puncture sites was controlled by gentle pressure and the spleen was returned to the abdominal cavity. The duodenum, liver and spleen were inspected prior to closing the abdominal incision.

G. Post-operative Care

Oral intake was withheld from all animals during the first

Plate VII: Intrasplenic implantation of re-suspended tissue fragments was achieved by direct injection into the splenic pulp using a 15 or 16-gauge needle.

post-operative day and only water was given for the second post-operative day. A regular kennel diet was resumed on the third day, supplemented with 4-6 capsules of pancreatic enzyme replacement (Cotazyme, Organon Canada Ltd, West Hill, Ontario). Five percent dextrose in normal saline, 25 ml/kg, was administered subcutaneously on the first and second post-operative day, and each animal received an intramuscular injection of 1.0 ml of Pen-Di-Strep (rogar/STB, London, Ontario) antibiotic solution concomitantly.

Dogs in the apancreatic control group (APC) received no additional medication. All transplanted animals received intermediate-acting NPH insulin (Connaught Laboratories Limited, Willowdale Ontario) 0.2-0.5 Units/kg, according to blood glucose determinations made the previous day. Insulin was administered subcutaneously each morning for 12 days.

H. Post-operative Observations

Post-operatively, the weight, general condition and fasting serum glucose levels of each of the apancreatic animals were recorded daily. The same parameters were measured in the transplanted groups daily for three weeks and three times each week thereafter. Intravenous glucose tolerance tests were performed on the third post-operative day in the pancreatectomized control group (APC) and at two and six weeks after surgery in the transplanted groups (CD, NCD). Mean blood glucose levels at timed intervals and their natural logarithms were plotted and k values obtained. Serum insulin levels were also determined, and insulin response curves

were constructed. Post-operative survival in days was recorded for each animal, and the mean survival and standard error of the mean was calculated for each group of dogs.

All animals were studied until they demonstrated severe clinical and biochemical signs of diabetes mellitus, necessitating sacrifice, or until the alloted time period for the project had elapsed. Three animals developed superficial wound infections. The wounds were cleaned with a solution containing chlorhexidine gluconate, irrigated with normal saline and painted with gentian violet solution. Postmortem examinations were performed and the following observations made: general condition of the animal, condition of the abdominal wound, any abnormality of stomach, duodenum or liver related to surgery, and the state of the spleen. Samples of any abnormal organs were taken and the entire spleen was sectioned transversely. Gross and microscopic examination of the splenic tissue was carried out to identify islet tissue in the spleen. Tissue was fixed in formalin and stained with hematoxylin and eosin as well as Gomori's aldehyde-fuchsin with Mallory's trichrome counterstain, to aid identification of islets. Preparation of histology slides was carried out in the Students' Laboratory, Department of Pathology, University of Alberta Hospital and the Histology Laboratory, Surgical-Medical Research Institute, University of Alberta.

I. Statistical Analysis

All results were expressed as mean ± SEM. Analysis of mean

serum glucose levels for each group was performed using Students' t-test for unpaired data. Mean serum insulin values were compared using analysis of variance. Differences were significant when $p < 0.05$ unless otherwise indicated.

A schematic summary of the experimental method is presented on the following page.

Outline of Experimental Method

Preoperative IVGTT and insulin response curves, normal controls (n=12)

APC Group (n=7)	CD Group (n=7)	NCD Group (n=7)
Total pancreatectomy	Total pancreatectomy	Total pancreatectomy
	Hand mincing	Hand mincing
	Collagenase digestion	
	Intrasplenic implantation	Intrasplenic implantation
	Exogenous insulin	Exogenous insulin
Postoperative IVGTT and insulin response: 3 days	Postoperative IVGTT and insulin response: 2 weeks, 6 weeks	Postoperative IVGTT and insulin response: 2 weeks, 6 weeks
Postmortem examination	Postmortem examination	Postmortem examination

Statistical Analysis

V. Experimental Results

All 21 animals operated upon survived during the immediate preoperative period. One animal developed vasomotor collapse and respiratory distress after the skin had been prepped with iodine surgical scrub solution. Resuscitation with subcutaneous adrenalin (0.5 ml of 1:1000 solution), intravenous fluid and assisted ventilation was successful, but the surgery was not performed and the animal was excluded from the study.

Wound infection was encountered in three animals, two from the APC group and one from the NCD group. Each of the dogs was a short-term survivor and was clinically severely diabetic. Wound dehiscience did not occur. One dog in the APC group developed bronchopneumonia and succumbed on the sixth post-operative day. Another animal from the NCD group died as a result of gastrointestinal bleeding.

The time required for tissue preparation and implantation in the NCD group was 56 $\pm$ 10 minutes and in the CD group 51 $\pm$ 12 minutes. The time interval varied directly with the amount of tissue processed and the number of injections required to implant the prepared fragments. Approximately five minutes of additional mincing was required in the NCD group before injection through the 15-gauge needle was possible.

Thirty-nine to 55 ml of settled tissue were available for intrasplenic implantation (2.4 ml/kg body weight). Subjectively,

collagenase treated tissue was easier to inject throught a 15-gauge needle, and could readily be implanted using a 16-gauge needle. Without collagenase, more repeated injections and often additional mincing were required. A 16-gauge needle generally could not be used for splenic implantation of tissue not treated with collagenase. Numerous pieces of tissue identified as duct and vascular elements were difficult to mince and had to be picked by hand from the tissue being chopped.

Islets were identified by microscopic examination of the prepared tissue fragments for both of the transplant groups prior to splenic implantation. Islets were generally noted to be encircled by exocrine tissue (Plates V & VI on page 26).

During splenic implantation tissue fragments could be seen percolating under the splenic capsule for several ml beyond the line of injection. Frequently the injected tissue remained clustered along the injection tract and was visible beneath the splenic capsule. In four animals, tissue fragments were seen travelling in small venous tributaries in the hilum and into the splenic vein. Subsequently, irregular dark areas less than five ml in greatest diameter were noted on the surface of the liver, clustered mainly about the porta hepatis. Similar hepatic changes were noted in three other dogs (2 NCD, 1 CD) in which venous embolization was not observed. The lesions on the surface of the liver were not seen at the time of postmortem examination and histologic sections of liver taken from the porta hepatis and anterior surface did not identify embolized fragments of pancreatic tissue.

On one occasion, the dark areas on the liver surface had disappeared prior to abdominal wound closure.

Survival in the apancreatic control group was 15.6 ± 4.4 days. Fasting hyperglycemia (serum glucose > 150 mg/dl) was present from the first post-operative day until the animals expired or were sacrificed. Fasting serum glucose levels ranged from 254 to 360 mg/dl, with a mean of 275 ± 11 mg/dl. Weight loss of 12 - 25% per week occurred, with the greatest loss occurring in animals which survived for the longest period post-operatively. In addition to weight loss, all animals displayed hyperphagia, marked thirst and polyuria in the early post-operative period. Four of seven animals were sacrificed because of severe lethargy and an inability to stand or feed.

Transplanted animals received subcutaneous insulin for the first 12 days post-operatively. Serum glucose levels were maintained between 60 and 200 mg/dl with 0.2 to 0.5 units/kg of NPH intermediate-acting insulin. During this period all the animals were active, had normal appetites, and stool frequency and character were similar to the pre-operative state. Weight loss of 0 to 5% per week was noted. After insulin therapy was withdrawn, the animals in both transplanted groups demonstrated progressive weight loss of 4 to 7% of body weight per week except two dogs in the CD group which had weight loss rates of 1.5 to 3.0% per week.

Mean serum glucose levels during IVGTT for control and transplanted groups at two and six weeks post-transplantation are presented

in Table I. Values for mean serum insulin levels during IVGTT for all groups are presented in Table II.

With the cessation of insulin therapy, all the animals in the CD and NCD groups, except the two noted above, developed persistent fasting hyperglycemia to levels similar to that seen in the APC group. Mean fasting glucose levels were 280 ± 27 mg/dl and 304 ± 14 mg/dl in the CD and NCD groups respectively, two weeks after transplantation, and 322 ± 14 mg/dl and 334 ± 10 mg/dl at six weeks. Survival in the NCD group was 24.5 ± 4.2 days, and was not significantly different than survival in the APC group ($p = 0.161$).

In the CD group, survival was 43.3 ± 11.4 days, and this was significantly greater than in the APC group ($p < 0.05$). Two dogs in this group survived 79 and 92 days and demonstrated fasting normoglycemia for 13 and 48 days respectively after insulin had been stopped, and their rates of weight loss were less than those of either the NCD group or the other dogs in the CD group.

The glucose tolerance and insulin response curves for normal and apancreatic control groups are depicted in Figure 1 and Figure 2. The glucose tolerance curves for the NCD and CD groups did not conform to the normal curve at either two or six weeks after transplantation. The curves for the transplanted groups more closely resembled that of the APC group as shown in Figures 3 and 4. The calculated k values for the transplanted groups were almost identical to that of the apancreatic control group, indicating effectively there was an absence of functioning islet tissue in the transplant recipients (Figs. 5 and 6). Insulin response curves

Table I

Group	Time (minutes)						
	0	10	20	30	60	90	120
NC	69 ± 2	203 ± 12	158 ± 11	128 ± 13	84 ± 3	84 ± 5	86 ± 5
APC	275 ± 11	381 ± 12	361 ± 12	346 ± 13	334 ± 10	328 ± 16	320 ± 15
CD-2	280 ± 27	397 ± 27	336 ± 30	361 ± 32	339 ± 27	330 ± 26	325 ± 25
NCD-2	304 ± 14	380 ± 16	369 ± 17	336 ± 17	346 ± 15	336 ± 13	336 ± 16
CD-6	322 ± 14	545 ± 32	427 ± 18	344 ± 10	356 ± 12	359 ± 14	309 ± 11
NCD-6	339 ± 10	376 ± 14	372 ± 11	366 ± 8	357 ± 8	355 ± 7	351 ± 8

I. Serum glucose levels (mg/dl) during IVGTT, for normal control (NC) and apancreatic control (APC) groups, and collagenase digested (CD) and non-collagnease digested (NCD) groups at 2 and 6 weeks post-transplantation (mean ± SEM).

Table II

Group	Time (minutes)						
	0	10	20	30	60	90	120
NC	19 ± 4.8	69 ± 12	44 ± 7.8	31 ± 10	14 ± 1.8	11 ± 1.3	13 ± 2.7
APC	3.8 ± 1.5	3.6 ± 0.91	2.4 ± 0.85	4.4 ± 1.8	3.0 ± 0.53	4.6 ± 0.95	3.7 ± 0.51
CD-2	5.9 ± 1.6	5.4 ± 2.0	6.7 ± 2.9	5.9 ± 2.6	5.9 ± 2.2	5.2 ± 1.5	4.8 ± 2.1
NCD-2	12 ± 5.7	7.3 ± 3.0	6.0 ± 0.79	6.6 ± 1.8	4.6 ± 0.48	11.0 ± 5.5	8.4 ± 2.8

II. Serum insulin levels (μU/ml) during IVGTT, for normal control (NC) and apancreatic control (APC) groups, and collagenase digested (CD) and non-collagenase digested (NCD) groups at 2 weeks post-transplantation (mean ± SEM).

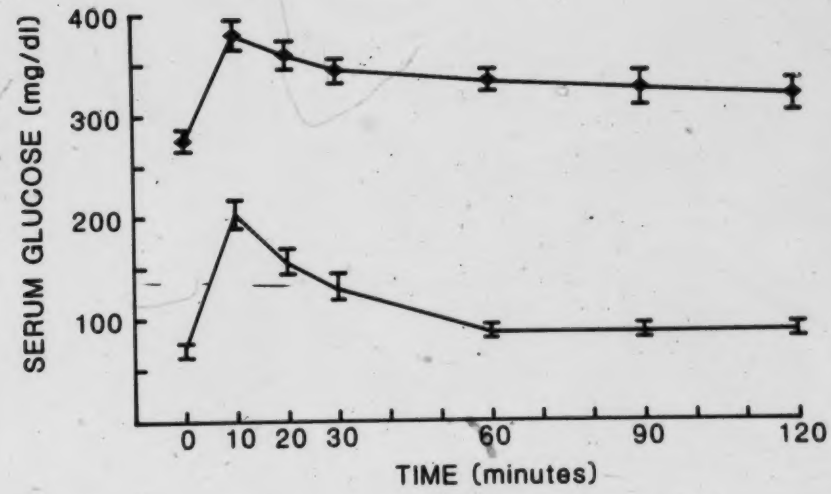

Figure 1: IVGTT Curves of Apancreatic Control (◆) and Normal Control (•) animals; mean ± SEM.

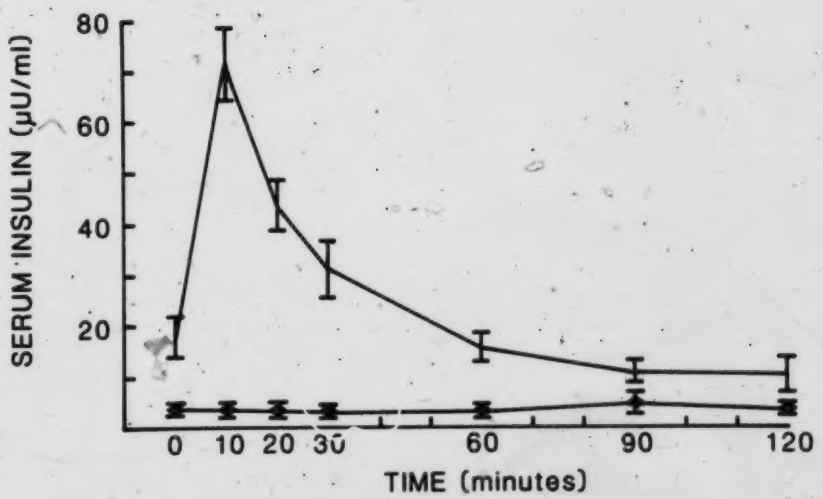

Figure 2: Serum insulin response curves of APC (n=7) and NC animals (n=12); mean ± SEM.

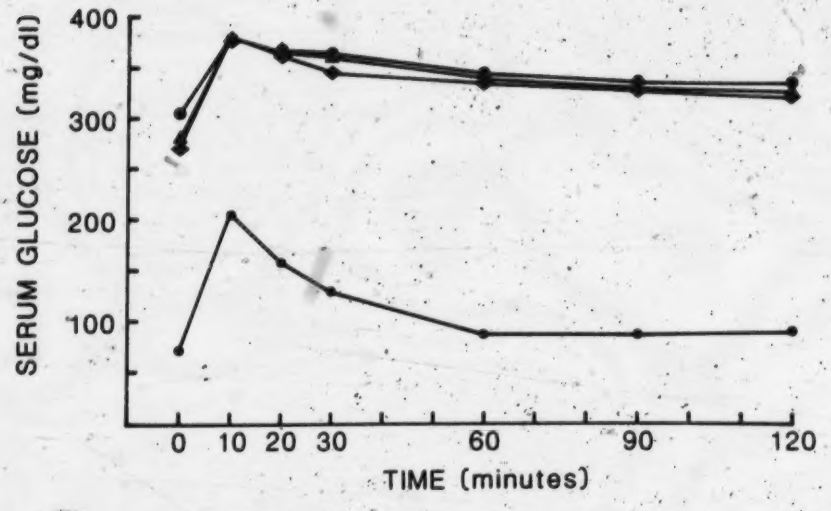

Figure 3: IVGTT curves of CD (▲) and NCD (●) groups at 2 weeks post-transplant, compared with APC (◆) and NC (▪) groups.

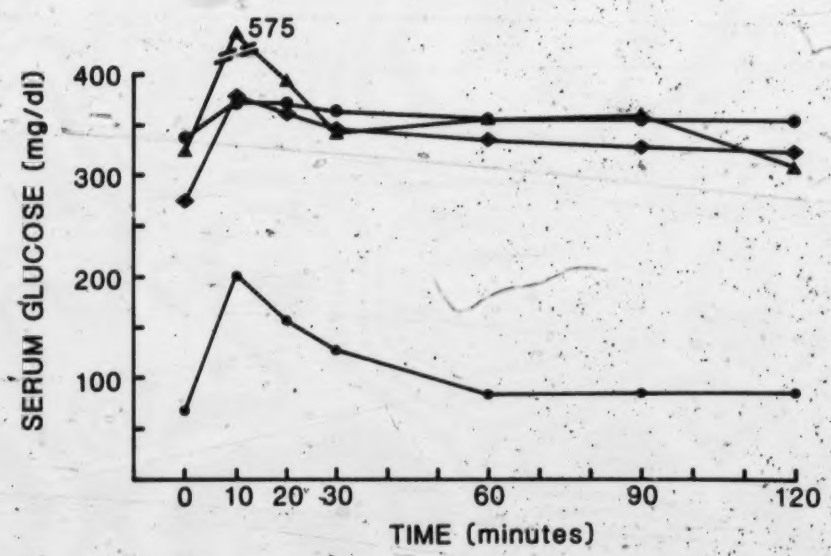

Figure 4: IVGTT curves of CD (▲) and NCD (●) groups at 6 weeks post-transplant, compared with APC (◆) and NC (▪) groups.

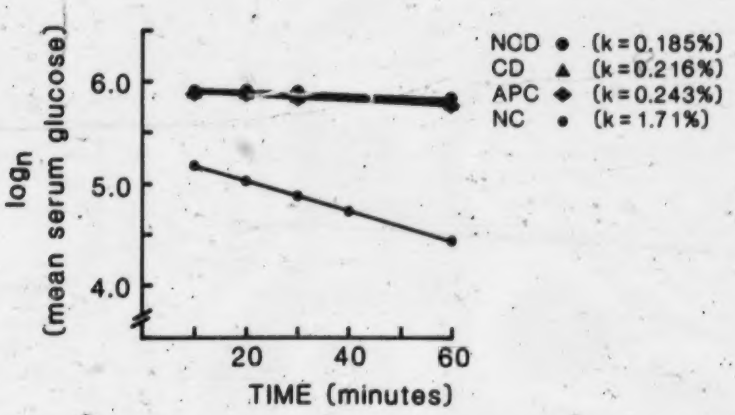

Figure 5: Glucose decay constants (k values) of CD (▲) and NCD (●) groups at 2 weeks post-transplant, compared with APC (◆) and NC (•) groups.

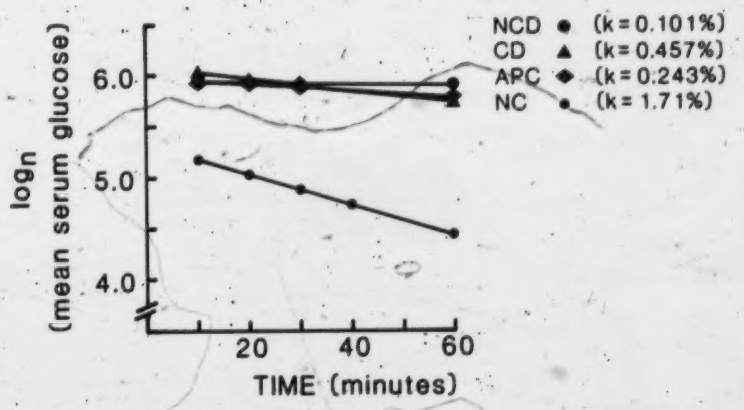

Figure 6: Glucose decay constants (k values) of CD (▲) and NCD (●) groups at 6 weeks post-transplant, compared with APC (◆) and NC (•) groups.

for both transplanted groups were abnormal and not significantly different from apancreatic controls (Fig. 7).

Two animals from the CD group had temporary periods of fasting normoglycemia with mean fasting glucose levels of 110 ± 10 and 100 ± 7.0 mg/dl. However, glucose tolerance curves were abnormal (Figs. 8 and 9) and insulin response was slow and of low magnitude during this time. Mean serum insulin levels were not significantly different from the APC group. The k values for these animals, although significantly different from the APC group, were still abnormal (Figs. 10 and 11). Both animals had to be sacrificed after hyperglycemia recurred because of severe cachexia.

Postmortem examination was performed on 19 of 21 animals studied. Two animals were inadvertently disposed of prior to examination. The following discussion represents a summation of postmortem findings from all of the animals examined.

The skin and fascia were well healed in all cases except for two of the three animals which had had wound infections. In those two cases the skin was incompletely healed with several areas of tissue granulation, but there was no evidence of suppuration. The fascia was well healed in each case. In the abdomen, fine adhesions were present about the duodenum in the area from which the pancreas was removed. The remainder of the abdomen was free of adhesions in all animals except one, in which the lower pole of the spleen was partially wrapped by an adherent leaf of omentum. The duodenum appeared grossly normal except in one of the NCD group

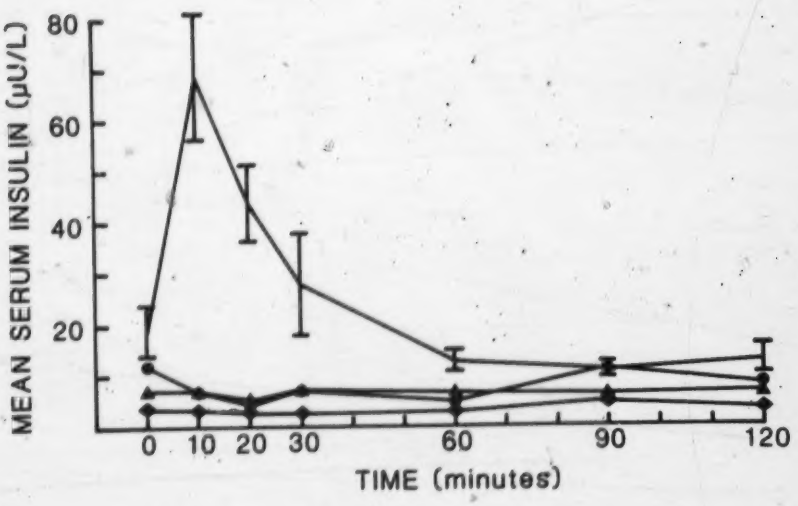

Figure 7: Serum insulin response curves of CD (▲) and NCD (●) groups at 2 weeks post-transplant, compared with APC (◆) and NC (•) groups.

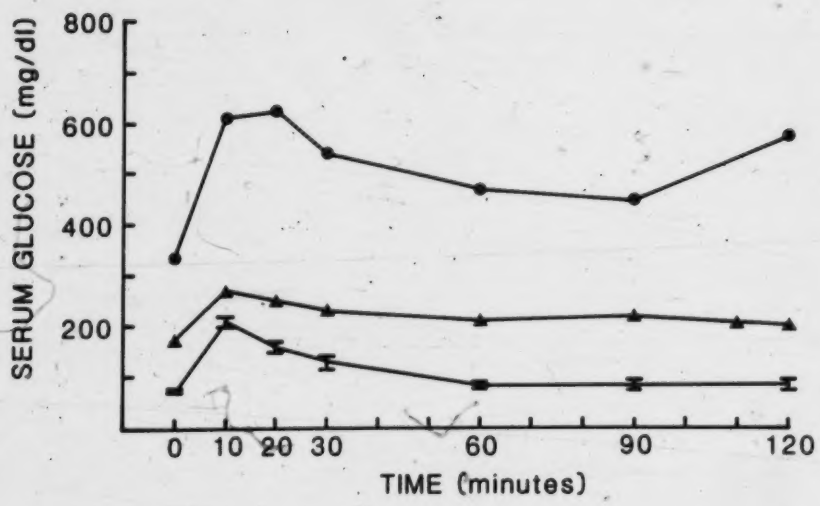

Figure 8: Comparison of IVGTT curves of CD animals A416 (●) and A715 (▲) that were temporarily normoglycemic at 2 weeks post-transplant, with the NC (•) group of animals.

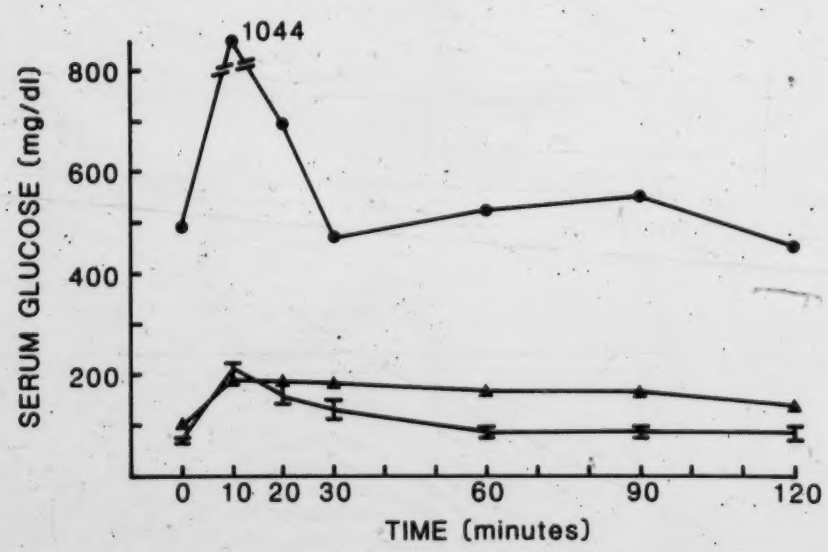

Figure 9: Comparison of IVGTT curves of normoglycemic CD animals A416 (●) and A715 (▲) at 4 weeks post-transplant; with the NC (•) group.

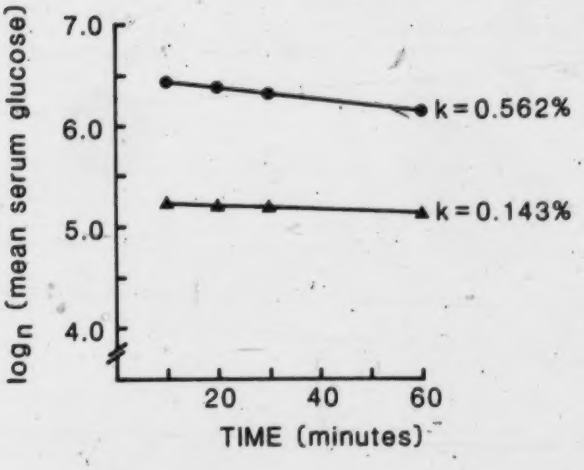

Figure 10: Comparison of k values of normoglycemic CD animals A416 (●) and A715 (▲) at 2 weeks post-transplant.

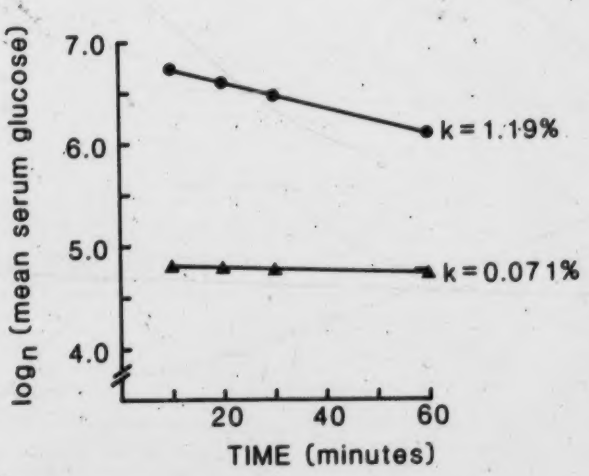

Figure 11: Comparison of k values of normoglycemic CD animals (A416 ● , A715 ▲) at 4 weeks post-transplant.

of animals which had had an episode of melena on the seventh post-operative day and subsequently expired on the 27th day as a result of profuse gastrointestinal bleeding. At autopsy, a circular ulcer four ml in diameter was located five cm from the pylorus on the mesenteric border. In the base of the ulcer a small blood-vessel was identified.

The spleen was shrunken to one-half or one-third the size noted at surgery. On the wrinkled splenic capsule of only seven of 19 specimens examined, small areas of hemorrhage or yellowish deposits were noted. On sectioning, these areas appeared to represent sites of implantation of pancreatic tissue and this was confirmed by histology. Pancreatic tissue fragments were readily found in microscopic sections of spleen made during the postmortem examination, usually adjacent to blood-vessels or splenic trabeculae (Plate VIII). Islets were identified in sections stained with aldehyde-fuchsin (Plate IX) and hematoxylin and eosin (Plate X), but were scarce. The predominant pancreatic tissue consisted of exocrine acini in a loose glandular arrangement. Granules within the exocrine cells were stained purple with the aldehyde-fuchsin, in addition to the islet cells. Several sections demonstrated an infiltrate of acute and chronic inflammatory cells about the auto-transplanted pancreatic tissue (Plate XI).

One animal in the APC group died on the fifth post-operative day and was clinically thought to have bronchopneumonia. At post-mortem exam. the lungs were heavy, wet and boggy; air-space consolidation with leukocytes and red blood cells were noted at microscopy.

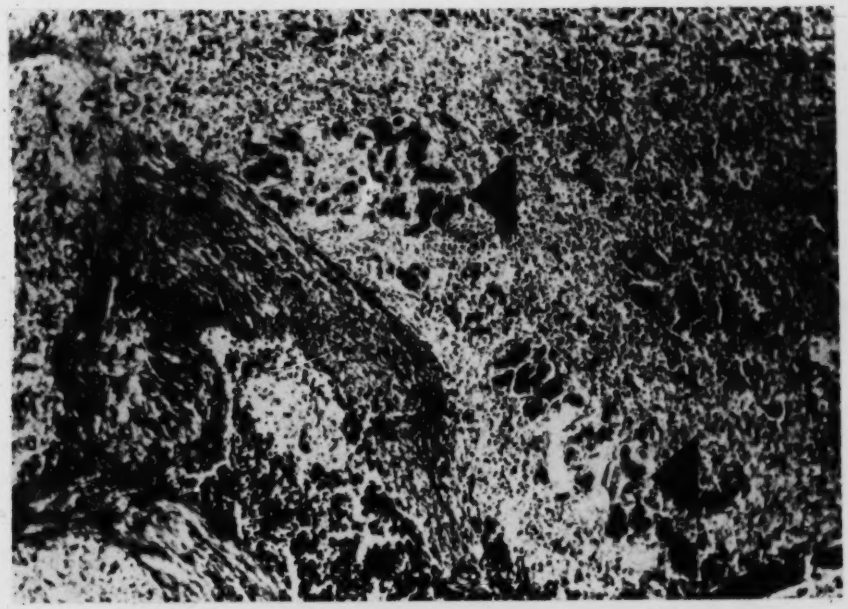

Plate VIII: In sections of spleen stained with aldehyde-fuchsin, endocrine tissue (arrows) was readily identified usually adjacent to blood-vessels or splenic trabeculae. (x 10)

Plate IX: Islets were difficult to find in the spleen. Aldehyde-fuchsin stained acinar and islet tissue purple. The glandular arrangement of the exocrine acini was obvious; islets were recognized as irregular clusters of variably stained cells (arrows). (x 100)

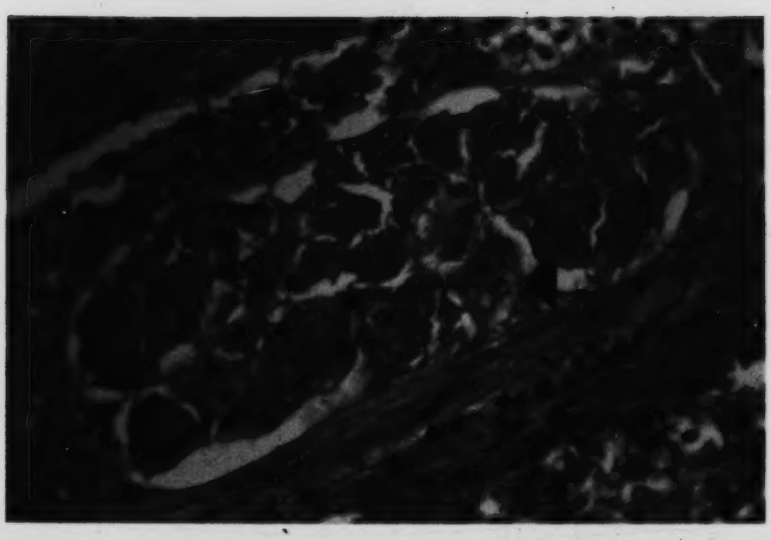

Plate X: Pancreatic tissue fragment located between splenic trabeculae, and containing a small islet (arrow). An infiltrate of inflammatory cells can be seen at the superior margin of the pancreatic tissue. (H and E x 100)

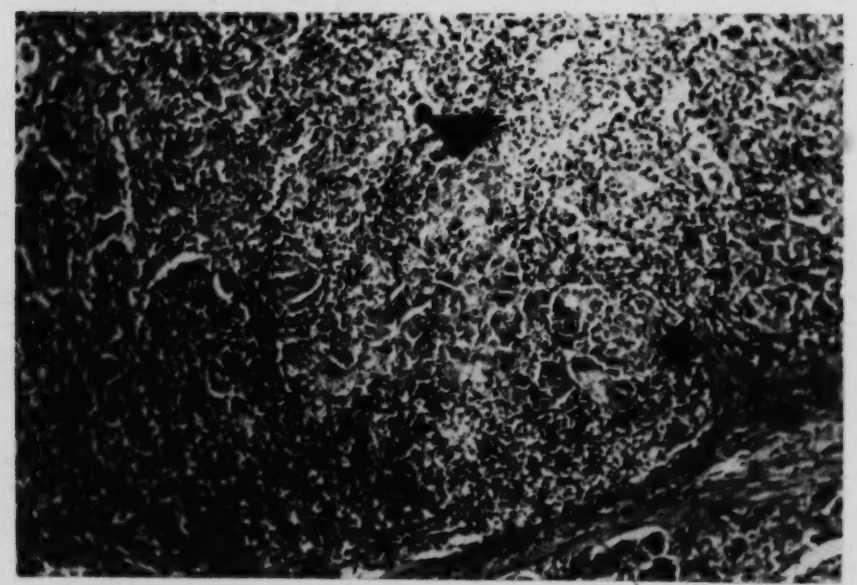

Plate XI: An intrasplenic pancreatic tissue fragment containing exocrine (large arrow) and endocrine (small arrow) elements is shown. A prominent inflammatory infiltrate is present in and about the autotransplanted tissue. (H and E x 40)

VI. Discussion of Results

All animals included in the study survived surgical manipulation and all were able to resume a normal diet by the third post-operative day. One dog developed an anaphylactic type of reaction just after the skin of the abdomen was cleansed with an iodine-containing solution. Resuscitation was successful but the animal was excluded from the experiment. Similar reactions have been noted when iodine solutions were used for peritoneal irrigation in dogs with experimentally induced intra-abdominal abscesses (J.D. Fischer, personal communication), although none of the other animals in our study reacted to the skin-prep solution.

Vascular injury to the duodenum, bowel obstruction, and bleeding or hematoma formation in the spleen were not encountered. One dog in the NCD group developed profuse gastrointestinal bleeding from a duodenal ulcer, resulting in the animal's death at four weeks post-transplant. The duodenum was normal in all other respects on gross examination, with no relation of the ulcer to the ligated pancreatic ducts. The ulcer appeared to have been peptic in origin, possibly as a result of stress. A second animal became ill with respiratory distress on the fourth post-operative day and died the following morning. Bilateral bronchopneumonia was confirmed at autopsy. One other animal, not included in our study but kennelled nearby, also demonstrated a similar clinical picture and was removed from the Vivarium.

Three superficial wound infections occurred post-operatively and all responded satisfactorily to cleansing and the application of gentian violet, used to promote wound re-epithelialization. A subcuticular skin closure with absorbable suture was employed to decrease local irritation and the dogs' inherent tendency to lick and chew at exposed sutures. A continuous absorbable suture was used for fascial closure of the abdomen, and wound dehiscience was not encountered.

Total pancreatectomy can be performed with minimal technical difficulty although care must be taken to preserve the duodenal branches of the pancreatico-duodenal vessels which course along the dorsal aspect of the mesenteric border of the duodenum (34). The anatomical relationships of the dog pancreas facilitate removal of all pancreatic tissue whereas in animals such as the pig, pancreatectomy is usually incomplete because of the intimate relationship of the uncinate process of the pancreas with the portal vein. Total pancreatectomy in dogs uniformly produces a diabetic state characterized by hyperglycemia, dehydration, weight loss and rapid clinical deterioration. In this study, the average survival was 15.6 ± 4.4 days after pancreatectomy; all animals in the APC group demonstrated a markedly abnormal IVGTT, and serum insulin levels were very low and unresponsive to dextrose infusion.

Post-operatively all animals received pancreatic enzyme supplements with their regular kennel diet. The use of enzyme supplements has been shown to control symptoms of impaired gastrointestinal absorption (35) and almost completely eradicate the severe diarrhea

and weight loss that presented significant handicap to long-term care and follow-up (58). Subjectively, stool frequency and character were not significantly altered in the dogs which received pancreatic enzyme supplements during the period of exogenous insulin administration. Specific testing for malabsorption was not carried out. All animals developed mild diarrhea and vomiting late in the course of their diabetes, however.

In addition to exocrine replacement, all transplanted animals received daily subcutaneous injections of intermediate-acting insulin for twelve days after surgery. Insulin has been used in an attempt to maintain normoglycemia in the early post-transplant period in clinical and experimental studies, and there has been some evidence that the survival of transplanted islets may be promoted in a normoglycemic milieu (8,24).

Normoglycemia was not observed in the two animals in the CD group until seven and nine days after insulin had been discontinued. There appeared to be a period of recovery or adjustment required by the islets in their new environment before endogenous insulin production and release resumed. This period of altered islet function was encountered by Mirkovitch and Campiche (58) and others (34,35), but exogenous insulin was not administered. In later studies by McEvoy et al (52) and Mirkovitch et al (60), it was suggested that an endocrine deficiency state was important for engraftment of transplanted islets. In spite of conflicting evidence regarding the use of exogenous insulin, the profound detrimental effects of diabetes observed in experimental animals and

human subjects after major surgery would logically dictate that exogenous insulin should be administered in the early post-operative period following pancreatic islet transplantation.

Upon the withdrawl of insulin, all but two of the transplanted animals began a clinical and biochemical course that was indistinguishable from that of the apancreatic controls. Fasting serum glucose levels, IVGTT curves and k values, and insulin response for both transplanted groups very closely approximated the values observed in the APC group, indicating the absence of an adequate number of functioning islets. That islets were in fact transplanted was confirmed by histological study of splenic tissue obtained immediately prior to splenic implantation; their functional capability after implantation was demonstrated in the two animals in the CD group that had short periods of fasting normoglycemia. Neither of these animals had normal k values however, and fasting normoglycemia lasted a maximum of seven weeks.

The use of dispersed pancreatic tissue fragments as a vehicle for islet transplantation was employed by Mirkovitch and Campiche (58) to avoid prolonged and difficult separation techniques that resulted in low islet yield from pancreases of larger mammals. This technique required implantation of a volume of tissue much larger than that of isolated islets utilized in experiments with rodents. When this volume of tissue was injected into the liver via the portal vein in the manner in which isolated rat islets were implanted, severe and often fatal complications developed (34,37,56). The spleen was found to be capable of accommodating the larger

volume of tissue (58) and successful transplantation of dispersed pancreatic tissue fragments has been demonstrated by several groups of investigators using a variety of implantation methods (34,36,58). Mirkovitch and Campiche reported success with retrograde venous embolization (58) while Kretschmer et al compared various methods of implantation and found direct implantation by multiple injections into the splenic pulp to be the most satisfactory (36).

In this study the spleen appeared to be a suitable site of implantation. Direct puncture and injection into the splenic pulp were uncomplicated and well tolerated. Although embolization of tissue fragments to the liver was observed, no major ill affects were evident, as the dark blue, presumably ischemic areas, on the surface of the liver were not evident at postmortem, and on one occasion, had disappeared before the abdomen was closed. Collateral circulation probably accounted for the lack of detrimental effects, as well as the fact that the volume of material embolizing to the liver was small.

The dispersion of the tissue fragments at each site of implantation may have been inadequate and did not permit nutrient diffusion to maintain viability of transplanted islets in the immediate post-transplant period, before revascularization had occurred. Injection via 15 or 16-gauge needles was difficult at times, and subcapsular "blots" of tissue were created when excessive force was required to accomplish injection. Collagenase-treated tissue was more easily injected and readily passed through the 16-gauge needle. Tissue which was not digested by collagenase usually could not be

injected through a 16-gauge needle, and probably reflected larger fragment size. Fragment size was not directly measured in these experiments. Mehigan et al (55) successfully autotransplanted to the spleen, dispersed pancreatic tissue fragments that passed through a 16-gauge needle, which has an internal diameter of approximately 0.8 mm. Our results were much less satisfactory using a similar technique, and it is reasonable to postulate that the use of smaller fragments of tissue would give better results. Downing and Scharp have developed a method for producing fragments and isolated islets with a diameter less than 0.5 mm (14,68). Normoglycemia is readily restored after transplantation of the small fragments, but the preparation of the tissue is significantly more complicated, and islet yield may be less than after conventional methods (68).

Pancreatic exocrine tissue was readily found at microscopy, but islets were very difficult to identify despite the use of special stains and multiple sections. Since we did not calculate islet yield or estimate the number of islets that were transplanted, the paucity of islets in sections of spleen obtained at post-mortem may have reflected a small number of islets that actually were transplanted. The inflammatory infiltrate noted in association with the pancreatic tissue may also have contributed to islet loss. An accurate assessment of islet yield prior to transplantation would have helped to clarify the reasons for the small number of islets identified in the splenic parenchyma.

The techniques of tissue preparation varied only in respect to the use of a 10 minute period of collagenase digestion. The time required for tissue preparation in each of the groups was not significantly different however, despite a 10 minute period of collagenase digestion in the CD group. This resulted from the difficulty encountered in injecting tissue in the NCD group through the 15 and 16-gauge needles and the extra time required for further hand-mincing. The laborious and inefficient nature of hand-mincing noted by other investigators (68,73) was apparent in our study, and the use of an automated mincing device would be expected to facilitate the initial steps in the preparation of dispersed tissue fragments, permitting rapid mincing of a larger volume of pancreatic tissue with a shorter ischemic time.

The use of collagenase to release cells from animal tissues was introduced by Moskalewski (63) and applied to islet isolation by Lacy and Kostianovsky (42). Initially used in the isolation of islets from rodent pancreas, collagenase digestion has become an integral process in the preparation of isolated islets and pancreatic tissue fragments using the more compact pancreas of larger animals, including human pancreas (71). However, exposure to collagenase has been demonstrated to result in a significant loss of islets (36) and, consequently, elaborate techniques have been developed to limit the duration of exposure to the collagenase (68).

Collagenase is an enzyme complex derived from the Clostridia species of bacteria. Its enzymatic constituents include clostripain, trypsin, non-specific protease and other unspecified enzymes, in

addition to collagenase itself. There is limited control over the make-up of collagenase produced by a culture of bacteria but, once produced and collected, its enzymatic composition can be assayed and the relative effectiveness in releasing cells from different types of tissue assessed. In this manner, collagenase can be typed and an individual lot can be said to have a measurable and consistent activity when used for a particular application. However, there is still wide variation in biologic activity among different lots of the same type of collagenase and the only practical method for determining the efficacy of a batch of collagenase is by trial and error (68,77). Once a specific lot has been found to adequately digest pancreatic tissue and release viable islets or produce islet-containing fragments, a supply of that lot can be obtained and stored for future use (68).

The Sigma type V collagenase used in these experiments had been shown to be effective in isolating rat islets, but had not been previously tested on canine pancreas. The presence of islets in the prepared tissue fragments was confirmed histologically prior to transplantation in both the CD and NCD groups, but assessment of islet number, viability, and functional capacity was not carried out. In this experiment, the biochemical and clinical response of the transplant recipients was used to measure the efficacy, or lack thereof, of the preparation techniques. The incorporation of a system which would permit in vitro measurement of insulin release by harvested islets coupled with a method of calculating islet yield, would permit elimination of tissue implantation and the sub-

sequent follow-up period. The assessment of techniques used to prepare islet tissue for transplantation would be simplified.

Kretschmer et al suggested that collagenase was responsible for a major proportion of islet loss or injury during tissue preparation (37). Using Worthington type IV collagenase (600 U/g of tissue), they demonstrated that a digestion period of 16 to 20 minutes was the most effective in producing successful pancreatic fragment transplants. Our study utilized Sigma type V collagenase (1000 U/g of tissue) and a period of 10 minutes was chosen to allow for the difference in type and concentration of collagenase. Although two animals which were transplanted with collagenase-treated tissue demonstrated temporary normoglycemia, the technique employed was generally inadequate. Additional experiments incorporating longer and shorter digestion times and varying concentrations of collagenase, may have permitted identification of an optimal duration of collagenase digestion.

Several methods of exposure to collagenase have been proposed for the preparation of pancreatic fragments. Direct intraglandular injection of room temperature collagenase, as described by Mirkovitch and Campiche (58), was found to be ineffective in our laboratory as well as that of others (36). Perfusion or distension of the pancreas via the ductal or venous system prior to mincing, was advocated by Kretschmer et al (36), and Downing et al (14). These investigators and others (34,55), have employed two periods of exposure to collagenase, before and after mincing, and have incorporated sophisticated systems of screens, filters and irrigation

to remove islets and microfragments from the collagenase as they are liberated. Using these methods, it was possible to restore fasting normoglycemia consistently after transplantation of the prepared pancreatic tissue (14,36,55).

Although the technique employed to prepare pancreatic tissue fragments in this series of experiments was largely unsuccessful, transient correction of hyperglycemia was achieved in two animals that had received tissue treated with collagenase. Support for the use of collagenase was provided by this limited success; but another pair of problems were also presented. Why did the functioning grafts fail after approximately two and seven weeks of fasting normoglycemia? Why were IVGTT curves and k values in the abnormal range despite normal fasting glucose levels? In their initial report in 1976, Mirkovitch and Campiche noted that all the transplanted animals had prompt and "almost normal" responses to glucose loading. Kolb et al (34) and Kretschmer et al (36) subsequently demonstrated that transplanted animals had consistently abnormal IVGTT curves and k values despite normal fasting glucose levels. The reversal of the diabetic state was incomplete, and the animals were in fact latent diabetics. Mirkovitch et al later confirmed these results in their laboratory (59).

Several explanations have been proposed to explain this rather disheartening finding. One suggests that simply an insufficient number of islets were transplanted, and that more tissue or different sources should be used; another suggests that the processing of the tissue and exposure to collagenase damages the islets, impairing

their function as well as decreasing their numbers. The small number of islets then become metabolically exhausted and, although fasting normoglycemia can be maintained, the response to a glucose challenge is impaired (34).

Islet fatigue may be a significant factor in latent diabetes observed after pancreatic fragment autotransplantation, but another site of islet loss must also be considered. Despite an absence of adverse histological changes observed in the splenic parenchyma by several investigators four to eight weeks post-transplantation (34,35,59), it may be possible that implanted pancreatic fragments initiate an inflammatory response which results in progressive islet attrition. In our experiments, an inflammatory infiltrate of uncertain significance was noted in sections of spleen containing pancreatic tissue. Histological examination of pancreatic tissue in splenic parenchyma six to 12 months after implantation may indicate that progressive fibrosis results in secondary deterioration of islet function, as noted by Idezuki et al after duct ligation of segmental pancreatic transplants (26).

Regardless of the mechanism of islet failure, however, the correction of the diabetic state after intrasplenic implantation of pancreatic tissue fragments has been demonstrated to be incomplete and the longevity of any initial correction has remained unknown (71). Improved methods of preparing and implanting pancreatic tissue fragments are required.

VII. Summary and Conclusions

Total pancreatectomy can be performed in the dog with low morbidity and mortality and a characteristic diabetic state is produced. Post-operatively, pancreatectomized animals also show signs of exocrine insufficiency: diarrhea and malabsorption can be corrected by supplementing the regular kennel diet with exocrine enzyme replacement. In the early post-operative period the signs and symptoms of diabetes were controlled by the administration of exogenous insulin. The use of insulin during this period is justified on clinical and biochemical grounds.

The objective of this project was to study a simple method of preparing dispersed pancreatic tissue fragments and to develop a working model of intrasplenic autotransplantation in the dog. Although we attempted to duplicate the work of other investigators by incorporating hand-mincing and a 10 minute period of collagenase digestion (36,55,58), the techniques employed in this experiment were probably oversimplified and as a result, ineffective. Hand-mincing is laborious and inadequate when used alone. Mechanical mincing also contributes significantly to islet loss, but some form of mechanical disruption appears to be necessary (35). The use of collagenase has been found to be an important element in tissue preparation, but it contributes to islet injury (68,71).

After reviewing the current literature and analyzing our own experiences, several observations and recommendations can be made

regarding the preparation of pancreatic tissue fragments.

1. The tissue should be chopped for as short a period as possible to limit islet injury.
2. An automated chopping device would shorten the ischemic time and permit processing of a larger volume of tissue.
3. Mincing may also be facilitated by preliminary retrograde venous or ductular distension of the intact gland with cold ($4^{\circ}C$) buffered salt solution which has been shown to initiate mechanical dissociation of endocrine and exocrine elements, apparently without damaging the islets (68).
4. Collagenase digestion must be controlled and liberated islets or tissue fragments must be protected or removed from the bulk of the tissue being treated to limit islet damage (14).
5. The optimal size of prepared fragments has not been determined but fragments approaching 0.5 mm in diameter would be expected to survive and function more efficiently after transplantation.
6. The final step in preparing pancreatic tissue fragments should be an evaluation of the product. Rather than following the course of a transplant recipient, in vitro assessment of islet yield and viability should be carried out so that modifications to the technique can be more readily applied.

7. The optimal technique for preparing pancreatic endocrine tissue for transplantation is not presently known.

Intrasplenic autotransplantation of dispersed pancreatic tissue fragments in the dog has evolved as an effective model for studying pancreatic islet transplantation. Endogenous insulin secretion and normoglycemia can be restored in totally pancreatectomized dogs. However, reversal of the diabetic state is incomplete and long-term function of the transplanted tissue has not been adequately demonstrated. Better techniques to prepare large numbers of viable islets or islet-containing fragments, are needed.

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